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ACCELERATED COMMUNICATION

N-Methyl-D-Aspartate Receptor Regulation of Uncompetitive Antagonist Binding in Rat Brain Membranes: Kinetic Analysis

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SUMMARY

N-Methyl-D-aspartate (NMDA) receptor ligands regulate the binding of uncompetitive antagonists in membranes prepared from rat brain. To determine the mechanism of this regulation, we examined the kinetics of the binding of the radiolabeled uncompetitive antagonist [^{3}H]*N*-(1-[thienyl]cyclohexyl) piperidine (TCP). Increasing concentrations of NMDA receptor agonists produced dose-dependent increases in the association and dissociation rate constants of TCP. The NMDA receptor antagonist amino phosphono valeric acid virtually abolished both the association and dissociation of TCP. Linear regression analysis detected a significant ($p < 0.001$) correlation between the effect of NMDA receptor ligands on the apparent association and dissociation rate constants. The most parsimonious explanation of the data is that NMDA receptor ligands regulate TCP binding by controlling access of TCP to a transiently accessible or "guarded" binding site located in the receptor-coupled ion channel. An increase in affinity or number of TCP binding sites is neither necessary nor sufficient to explain the potentiation of TCP binding produced by NMDA agonists. This finding validates the use of uncompetitive antagonist binding as a measure of the *functional* activation of the NMDA receptor-coupled ion channel in isolated membrane preparations.

The NMDA subtype of excitatory amino acid receptor is critically involved in several forms of neuronal plasticity (1, 2), anoxic and ischemic neuronal injury (3), and formation of synaptic connections in the developing nervous system (4). This receptor is coupled to a voltage-dependent cation channel permeable to Ca^{2+}, Na^+, and K^+ (5, 6). The receptor/channel complex also contains a strychnine-insensitive glycine receptor, activation of which increases the frequency of channel opening evoked by NMDA agonists (7). NMDA-evoked current can be blocked in a competitive manner by antagonists such as APV that displace NMDA from its receptor (8). NMDA evoked currents can also be blocked by UCAs such as phencyclidine, ketamine, and MK-801 (9, 10).

NMDA receptor agonists potentiate the binding of radiolabeled UCA to rat brain membranes (11–16); this potentiation is markedly enhanced by glycine (14, 16). Previous studies, conducted under presumed equilibrium conditions, have suggested that NMDA regulates either the affinity (11–13) or the number (14) of UCA sites. Recently, a third possibility has been raised by electrophysiological experiments in which both the development and relief of blockade of NMDA-evoked currents by UCA were shown to require the presence of NMDA agonists (9, 10). This suggested that UCAs produced the blockade by lodging within the lumen of the receptor-coupled channel and that the lodging and dislodging of the UCA required the channel to be in an open conformation. If this model of UCA blockade of NMDA evoked currents is accurate, then NMDA agonists may increase UCA binding in isolated membranes by increasing the proportion of time that the channel is in the open conformation, thereby allowing increased diffusion of radiolabeled UCA into otherwise inaccessible binding sites. This mechanism will be referred to as the "guarded receptor hypothesis," a term adopted from Starmer *et al.* (17, 18) who quantitatively described an analogous process in blockade of voltage-regulated sodium channels.

Kinetic binding experiments were used to investigate the mechanism by which NMDA receptor ligands regulate UCA binding. This approach allowed distinguishing among changes in receptor number, affinity, and accessibility.

Materials and Methods

Membrane preparation. Hippocampal membranes were prepared from male Sprague-Dawley rats weighing 300–375 g. Rats were killed by decapitation. The hippocampi were homogenized in 10 ml of ice-cold 50 mM Tris·HCl buffer containing 10 mM EDTA (pH 7.7 at 25°)

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; APV, amino-phosphono-valeric acid; TCP, *N*-(1-[thienyl]cyclohexyl)piperidine; UCA, uncompetitive antagonist; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; FG9065, 6-cyano-2,3-dihydroxy-7-nitroquinoxaline.

with a Brinkman Polytron (20 sec, setting 6). Homogenates of 10–20 hippocampi were pooled, separated into 10-ml aliquots, and centrifuged (37000 × *g*, 20 min, 4°). Supernatants were discarded and the pellets were homogenized in 50 mM Tris-EDTA buffer. After centrifugation the pellets were homogenized in 10 ml of buffer and quickly frozen by immersion in a dry ice/methanol bath. The membranes were thawed, homogenized, and centrifuged. The pellet was suspended in 5.0 mM Tris·HCl, (pH 7.7 at 25°, no EDTA), homogenized, and frozen as before. The membranes were stored (24 hr to 1 week) at −60° until used in a binding assay. On the day of assay the membranes were thawed, homogenized, diluted with 5 mM Tris·HCl to a volume of 40 ml of buffer per hippocampus, and centrifuged. The pellets were washed once more in 5 mM Tris·HCl (40 ml/hippocampus), resuspended in Tris·HCl (10–15 ml/hippocampus), and homogenized by Polytron immediately before use in the binding assay.

TCP binding assay. Specific TCP binding (2.5 nM) (difference in absence and presence of 1.25 μM nonradioactive TCP) was measured in 1 ml of a 5 mM Tris·HCl buffer (pH 7.7 at 25°). All incubations included 10 μM glycine unless otherwise specified. Protein concentrations ranged from 0.07 to 0.15 mg/ml. Reactions were terminated by vacuum filtration using a Skatron cell harvester and Skatron Filtermats (no. 7034). TCP binding to the filters was reduced by pretreatment with 5 ml of 0.075% polyethylenimine immediately before membrane filtration. After membrane filtration, the filters were rinsed for 10 sec with ice-cold buffer. Radioactivity was measured by liquid scintillation counting.

TCP association experiments were conducted by incubating hippocampal membranes with 2.5 nM TCP for varying periods of time before filtration. TCP dissociation experiments were conducted after first labeling the membranes by incubation with TCP. Dissociation of TCP commenced with the addition of 0.1 ml of buffer containing 12.5 μM nonradioactive TCP, for a final concentration of 1.2 μM nonradioactive TCP. Nonspecific binding did not vary as a function of time. In membranes incubated for 10 min in the presence of optimal concentrations of NMDA or glutamate, specific binding accounted for 80% of the total binding. Apparent association and dissociation rate constants were determined using nonlinear curve-fitting programs (19). All experiments have been replicated at least three times unless specified otherwise.

Analysis of kinetic binding data. A useful framework in which to characterize the interaction of ligands with transiently accessible (e.g., channel) binding sites is a modification of the "guarded" receptor model described by Starmer *et al.* (17, 18). In this model the interaction of a ligand and binding site can be described by the equation given below.

$$R + L \underset{FK_2}{\overset{FK_1}{\Leftrightarrow}} RL$$

R is the number of unbound sites, L is the concentration of unbound ligand, RL is the concentration of bound ligand, K_1 is the association rate constant, K_2 is the dissociation rate constant, and F is the fraction of time that the overall population of receptors are in an accessible conformation. In the traditional, continuously accessible, model of ligand-receptor interactions the factor F would equal 1. Alternatively, in the case of a ligand binding to a site within the lumen of the channel, complete closure of the channel would result in F approaching 0. In this case both ingress and egress of ligand from the binding site would be abolished. The terms FK_1 and FK_2 will be referred to as apparent association and dissociation rate constants, respectively. This model is a simplification of the model described by Starmer in that it assumes the factor F to apply equally to the association and dissociation reactions. Stated differently, channel activation is assumed to modify access and egress of the ligand equally.

This model allows testing of the hypothesis that NMDA regulates TCP binding by increasing access to a channel binding site rather than increasing the affinity or number of binding sites, because each possibility predicts a unique set of changes in the apparent association and dissociation rate constants (Table 1).

TABLE 1

Comparison of possible mechanisms of NMDA effects

This table compares possible mechanisms by which NMDA might increase TCP binding and the predicted outcomes of kinetic binding experiments.

Mechanism of increased UCA binding	Association rate constant (FK_1)	Dissociation rate constant (FK_2)	Apparent K_d (FK_2/FK_1)	Binding at steady state
Increased number of sites	No change	No change	No change	Increased
Increased affinity	Increased[a]	Decreased[a]	Decreased	Increased
Increased access to (and from) the site	Increased	Increased	No change	No change

[a] Increased affinity can theoretically be the consequence of an increase in the association rate constant, a decrease in the dissociation rate constant or both. It should also be noted that if either accessibility or egressibility of the ligand alone is modified this will be detected as a change in apparent affinity even though the true affinity of the binding site is not altered.

Results

Agonists of the NMDA receptor subtype selectively stimulated TCP binding under nonequilibrium conditions (Fig. 1). Glutamate and NMDA increased TCP binding in a dose-dependent manner. The highest concentrations of glutamate and NMDA resulted in a reduction of TCP binding, confirming previous results (13, 14). The stimulation was selective to the NMDA receptor subtype inasmuch as kainate and the selective quisqualate receptor agonist AMPA (20) did not increase TCP binding.

Glycine (10^{-5} M), while having minimal effects on TCP binding by itself, markedly potentiated the action of glutamate and NMDA. The most prominent effect of glycine was to increase the maximal response to NMDA and glutamate, rather than to shift the potency. In the presence of glycine, NMDA and glutamate produced approximately equivalent increases in TCP binding. However, the decreased response seen at high concentrations of NMDA and glutamate precluded precise comparisons of the maximal response.

To further characterize the receptor subtype involved in regulation of TCP binding, the effects of selective antagonists were examined. Glutamate potentiation of TCP binding was attenuated by the NMDA receptor antagonist D-APV, both in the presence and absence of added glycine. By contrast, the selective quisqualate/kainate receptor antagonist FG 9065 did not reduce glutamate-dependent TCP binding at concentrations selective for these receptors (10^{-5} M). The enhancement of TCP binding by 10^{-3} M kainate was blocked by APV (10^{-4} M) (data not shown), suggesting that high concentrations of kainate activated NMDA receptors. The TCP binding detected in the absence of added glutamate and glycine was almost abolished by APV (10^{-4} M), thereby suggesting the presence of small amounts of glutamate or aspartate despite extensive washing of the membranes as reported by Murphy *et al.* (20).

To determine the mechanism by which NMDA receptor ligands regulate TCP binding, kinetic binding experiments were performed. NMDA produced dose-dependent increases in the association rate of TCP binding (Fig. 2). However, as equilibrium was approached, TCP binding became equivalent regardless of NMDA concentration. The finding that NMDA had no effect on TCP binding at equilibrium was not the consequence of inactivation of the NMDA receptor or the gradual accumulation of NMDA agonists in the incubation media inasmuch as, even after this long incubation, the rate of dissociation of

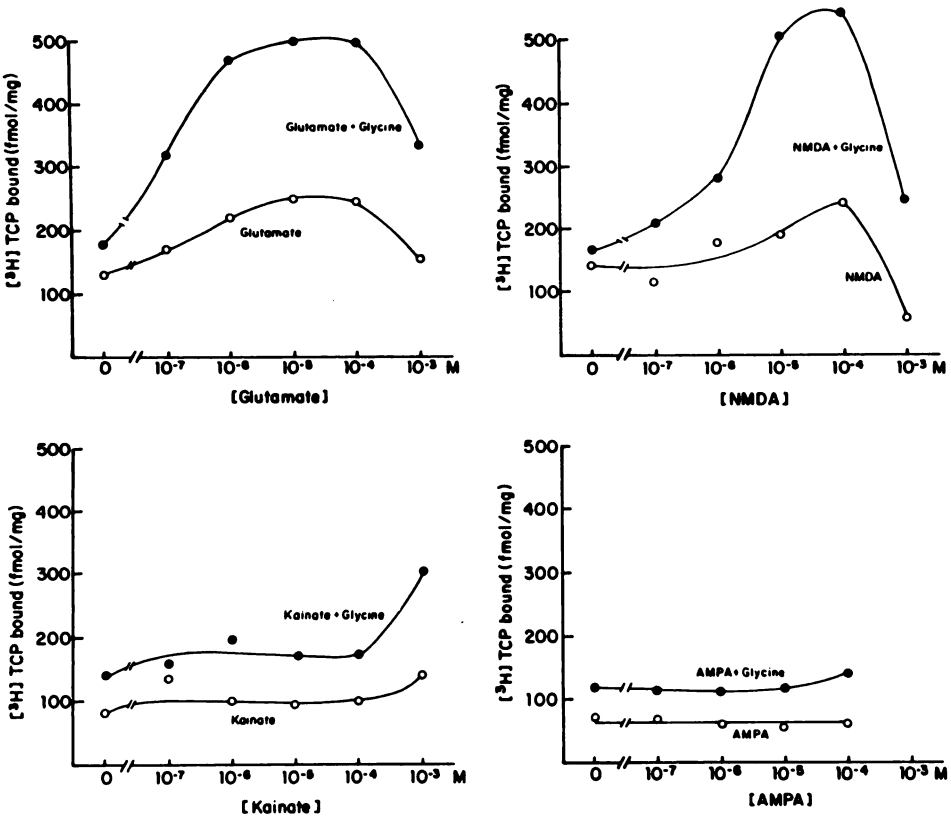

Fig. 1. Effect of NMDA, L-glutamate, AMPA, and kainate on specific TCP binding in the absence and presence of 10 μM glycine. Values are specific TCP bound after a 10-min incubation (nonequilibrium). Each data point represents the mean of triplicate measures. Each experiment has been replicated at least twice except the AMPA curves, which have been replicated once.

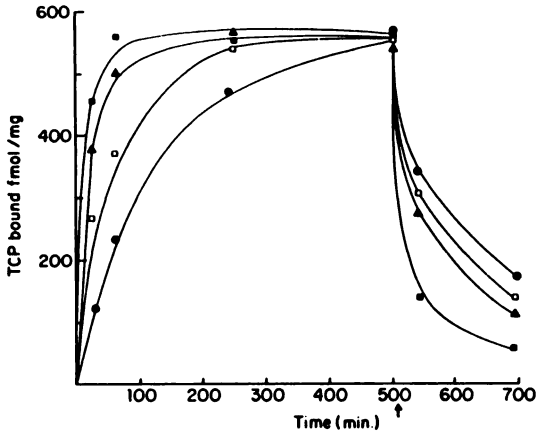

Fig. 2. Effect of NMDA on the association and dissociation of TCP. Values are specific TCP binding (mean of duplicates). *Arrow* marks the addition of nonradioactive TCP. Association component has been replicated twice. Dissociation component has been replicated once. There was no statistically significant difference in TCP binding at the 500-min mark of the incubation. ●, Basal; □, 1×10^{-6}; ▲ 3×10^{-6}; and ■, 1×10^{-4} M NMDA.

TCP (upon addition of nonradioactive TCP) was dependent upon the concentration of NMDA originally added to the incubation.

To quantitatively compare the effects of NMDA on the apparent association and dissociation rate constants, the initial portions of the association and dissociation curves were analyzed using nonlinear curve-fitting programs. NMDA produced a dose-dependent increase in the observed apparent association rate constant (Fig. 3) (visualized as a dose-dependent increase in the slopes of the natural log (ln) transformation of the association curves). The lines generated by the ln-transformed data were not linear, indicating that the observed association rate constants determined by curve fitting of the raw data are underestimates of the initial rate constant. NMDA also produced dose-dependent increases in the dissociation rate of TCP binding (Fig. 4). The nonlinear curve-fitting analysis indicated that NMDA produced increases in the apparent dissociation rate constants. As in the case of the association curves, the ln transformations of the dissociation data were nonlinear and thus the calculated rate constants are also underestimates of the initial dissociation rate constant. In a separate series of experiments, the effect of APV on the association and dissociation of TCP was determined in the absence of added NMDA and glycine. In these experiments APV (10^{-4} M) markedly reduced both the association and dissociation of TCP (Fig. 5). Linear regression analysis revealed a statistically significant correlation in the effects of NMDA receptor ligands on the apparent rate constants (Fig. 6). Because the NMDA receptor ligands produced equivalent changes in both rate constants, there was no appreciable alteration in the kinetically determined K_d (Table 2).

Discussion

Our principal findings include the following: 1) TCP binding is regulated by agonists and antagonists of the NMDA receptor but not by agonists or antagonists of the kainate and quisqualate receptors; 2) glycine selectively potentiated the effect of NMDA and glutamate on TCP binding but did not modify the effects of selective concentrations of kainate or the quisqualate receptor agonist AMPA; 3) NMDA, although have no effect on TCP binding at steady state, produced dose-dependent and quantitatively similar increases in both the association and

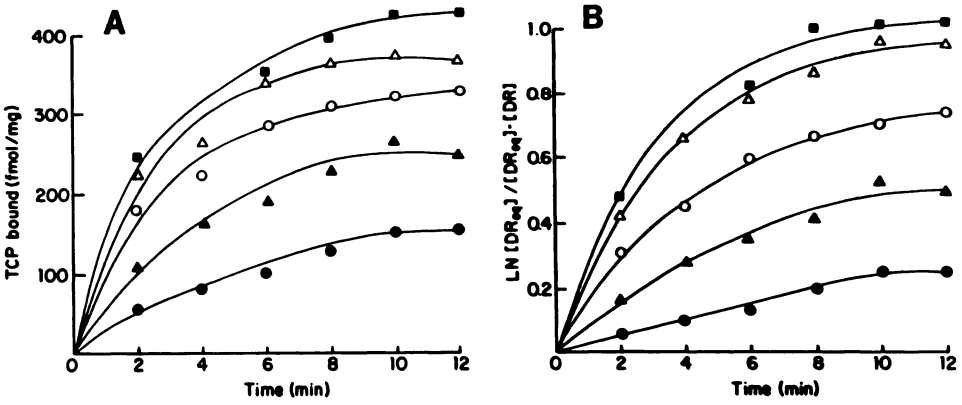

Fig. 3. A, Association of TCP with its binding site in the presence of varying concentrations of NMDA. ●, Basal; ▲, 3 × 10^{-6}; ○, 1 × 10^{-5}; △, 3 × 10^{-5}; and ■, 1 × 10^{-4} M NMDA. B, ln transformation of the association curves. Slopes of these lines provide an estimate of the apparent observed association rate constant.

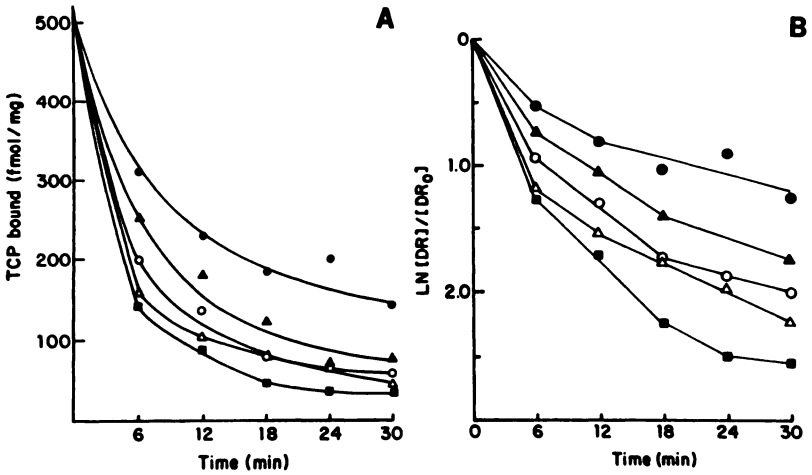

Fig. 4. A, Dissociation of TCP from its binding site in the presence of varying concentrations of NMDA. ●, Basal; ▲, 3 × 10^{-6}; ○, 1 × 10^{-5}; △, 3 × 10^{-5}; and ■, 1 × 10^{-4} M NMDA. B, ln transformation of the dissociation curves. The slopes of the ln transformed data give an estimate of the apparent dissociation rate constant. Membranes were preincubated with TCP for 2 hr in the absence of added amino acids, before initiation of the dissociation reaction.

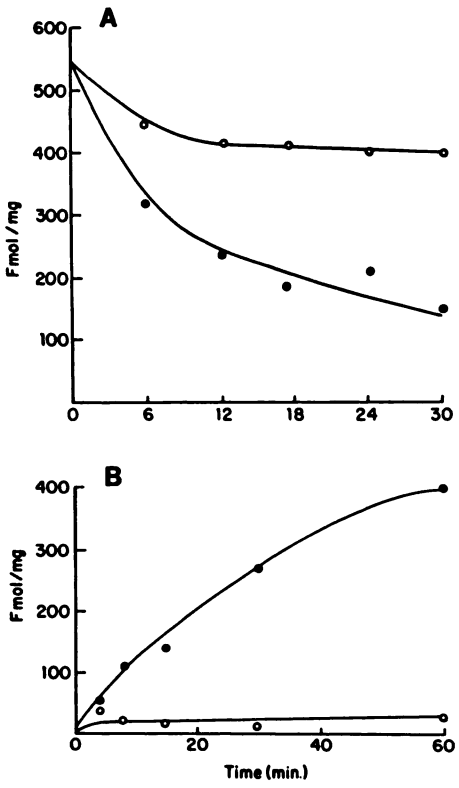

Fig. 5. A, Dissociation of TCP in the absence (●) and presence (○) of APV (10^{-4} M). B, Association of TCP in the absence (●) and presence (○) of APV (10^{-4} M).

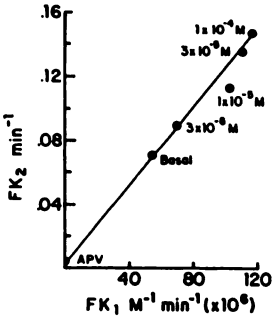

Fig. 6. Correlation of the apparent association (FK_1) and dissociation (FK_2) rate constants in the presence of varying concentrations of NMDA. The linear regression was statistically significant ($p < 0.001$).

dissociation rate constants of TCP binding; and 4) the NMDA antagonist APV virtually abolished both association and dissociation of TCP.

The effect of NMDA receptor ligands on the apparent rate constants was used to distinguish among three mechanisms by which NMDA receptor ligands may regulate TCP binding. The hypothesis that NMDA increases TCP binding by increasing the number of UCA binding sites (14) is not supported by the data because, alone, an increase in binding site number will not modify the association and dissociation rate constants. Moreover, there was no effect of NMDA on TCP binding under equilibrium conditions. The hypothesis that NMDA enhances UCA binding by increasing the affinity of the UCA binding site (11–13) is also not supported by these data because the equiv-

TABLE 2

Effect of NMDA on apparent rate constants

NMDA produced a dose-dependent increase in the observed association rate (FK_{obs}), the apparent association rate (FK_1), and the apparent dissociation rate (FK_2) constants of TCP binding ($p < 0.01$ by trend analysis) but no change in the kinetically determined K_d. Calculation of rate constants (± standard error) was based on the first 12 min of both the association and the dissociation reaction. To calculate a K_d from these rate constants it is necessary to assume that: 1) the factor F is identical in the association and dissociation reactions (so that it can be factored from the equation FK_2/FK_1); 2) TCP is binding to a single receptor population. Because it is unclear that these conditions are met, the K_d is labeled "apparent K_d." This value is presented for comparison with previously published values and to demonstrate that NMDA has no dose-dependent effect on the ratio of the rate constants.

NMDA	FK_{obs}	FK_1	FK_2	Apparent K_d
M	min^{-1}	$10^{+6}\ M^{-1}\ min^{-1}$	min^{-1}	nM
Basal	0.21 ± 0.05	54.8 ± 13.2	0.07 ± 0.01	1.27
3×10^{-6}	0.26 ± 0.03	69.6 ± 7.9	0.09 ± 0.02	1.29
1×10^{-5}	0.37 ± 0.02	103.6 ± 4.2	0.11 ± 0.03	1.10
3×10^{-5}	0.42 ± 0.03	112.4 ± 6.7	0.14 ± 0.08	1.21
1×10^{-4}	0.45 ± 0.01	118.8 ± 1.9	0.15 ± 0.03	1.25
1×10^{-4} D-APV	0.01 ± 0.01	0.0 ± 0.0	0.01 ± 0.01	NA*

* Not applicable.

alent changes in both rate constants resulted in no change in the kinetically determined K and because there was no effect of NMDA on TCP binding under equilibrium conditions. The data are most consistent with the hypothesis that NMDA receptor ligands simply regulate the accessibility of TCP to a binding site in the NMDA receptor-coupled channel. If TCP binds within the NMDA receptor-coupled channel, then agonist occupancy of the NMDA receptor should increase the total channel open time and thereby produce parallel increases in the rates of both ingress and egress of the ligand. Together, this would result in *identical* changes of both the apparent association and dissociation rate constants, because the factor F is common to each. The data support this mechanism because there was a direct linear relationship between the NMDA- and APV-evoked changes in the association and dissociation rate constants (Fig. 6).

Together these kinetic analyses are qualitatively and quantitatively consistent with the guarded receptor hypothesis of NMDA regulation of UCA binding and coincide with electrophysiologic studies. This interpretation is further supported by recent kinetic and equilibrium analyses of the effects of NMDA and glycine on TCP binding in membranes derived from rat cortex (21). We recognize that more complicated alternatives cannot be excluded. However, changes in affinity or number of TCP binding sites are neither necessary nor sufficient to explain these data.

The disparity between the findings presented here and previous investigations reporting that NMDA receptor ligands alter the affinity or number of TCP binding sites likely arises from the differences in experimental approach. Previous investigations of NMDA regulation of UCA binding have used presumed equilibrium binding conditions. The results of the experiments presented here indicate that the assumptions used in conducting these equilibrium binding experiments may not be valid and therefore the conclusions may not be supported by the data.

Several significant problems may confound the use of equilibrium binding experiments in investigating NMDA regulation of UCA binding. Analysis of binding data by the methods of Scatchard and others require the assumption that equilibrium of the ligand-receptor interaction has been reached. Under conditions of infrequent channel opening, such as in the case of no NMDA receptor activation, the factor F approaches zero and the half-life of the association reaction ($0.693/FK_{obs}$) approaches infinity, making establishment of equilibrium nearly impossible. In principle, in the presence of NMDA and glycine, a steady state of channel open time would allow equilibrium of UCA binding and estimation of K_d and B_{max} values. However, if a given manipulation of the receptor/channel complex (e.g., addition of an ion) resulted in an apparent change in the affinity of the TCP binding site, this change could be due to an alteration in channel-regulated diffusion of ligand rather than a change in actual affinity of the binding site (18). Demonstration that ingress and egress of UCA were not differentially modified would be a necessary prerequisite before concluding that the UCA binding site itself had been altered. An additional concern in estimating the K_d and B_{max} of UCA binding sites from equilibrium data is the difficulty in distinguishing radiolabeled UCA that is actually bound to the binding site from UCA that is not bound but is merely trapped in the closed channel. Finally, because TCP is only relatively selective for NMDA channels, the high concentration of TCP used in equilibrium experiments may result in appreciable amounts of TCP binding to other sites, such as the haloperidol-sensitive site (22).

The nonlinearity of the ln transformations of the association and dissociation data deserves comment. This may be a consequence of TCP binding to multiple sites with varying affinity. However, if TCP is binding to multiple sites in this preparation, then these sites are likely coupled to the NMDA receptor inasmuch as APV could almost completely eliminate the association and dissociation reactions. An alternative explanation is that there is heterogeneity in the opening frequency of the NMDA receptor-coupled channels; that is, in the presence of equivalent concentrations of NMDA and glycine, some channels spend a greater portion of their time in the open conformation than do others. This interpretation is consistent with the nonlinearity of the ln transformations of both association and dissociation data as well as the APV sensitivity of all components of the association and dissociation reactions. Regardless of the mechanisms underlying the nonlinearity of the ln transformation of the data, these results do not invalidate the basic contention that NMDA ligands regulate TCP binding by controlling access of the ligand to its binding site(s).

The finding that NMDA regulates TCP binding by controlling access of the ligand to the channel binding site, rather than by altering the number or affinity of the TCP binding sites, validates the use of TCP binding as a biochemical marker of activation of the NMDA receptor coupled ion channel. Appropriate use of UCA binding should facilitate analysis of the mechanisms controlling interaction among the components of this macromolecular complex. Such interactions are potential sites for regulation of function of the NMDA receptor/channel complex under physiologic conditions such as the formation of memory or the formation of synaptic connections in the developing nervous system. Modification of these interactions might also contribute to pathologic conditions including epileptogenesis, ischemic neuronal injury, and others. Finally, regulation of UCA binding kinetics provides a simple biochemical assay for dinstinguishing agonists from antagonists of both NMDA and glycine receptors.

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